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Characterization of *Beauveria bassiana* (Ascomycota: Hypocreales) isolates associated with *Agrilus planipennis* (Coleoptera: Buprestidae) populations in Michigan

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ABSTRACT

Earlier research in Michigan on fungal entomopathogens of the emerald ash borer (EAB), a major invasive pest of ash trees, resulted in the isolation of *Beauveria bassiana* from late-instar larvae and pre-pupae. In the present study, some of these isolates were characterized and compared to ash bark- and soil-derived isolates to determine their reservoir and means of infecting immature EAB. Genetic characterization using seven microsatellite markers showed that most of the EAB-derived strains clustered with bark- or soil-derived strains collected from the same site, indicating the indigenous nature of most strains isolated from EAB. More soil samples contained *B. bassiana* colony forming units than bark samples, suggesting that soil serves as the primary reservoir for fungal inocula. These inocula may be carried by rain splash and air current from the soil to the lower tree trunk where EAB may become infected. Additionally, inocula could come from infected EAB or other insects infesting ash trees. Bioassay of EAB adults, exposed by dipping in conidial suspensions (10^6 conidia/ml), showed all five representative strains with comparable virulence to the commercial strain GHA. These data demonstrate that indigenous strains of *B. bassiana* have potential for use as control agents against EAB and suggest that fungal inocula applied to ash trunks may prove viable for controlling EAB in the field.

1. Introduction

Agrilus planipennis Fairmaire (Coleoptera: Buprestidae), the emerald ash borer (EAB), is an invasive pest from northeastern Asia that causes mortality of ash trees (*Fraxinus* spp.) in North America. Adults feed on ash foliage and lay eggs in bark crevices or between bark layers. Eclosing larvae bore into the bark and feed on ash phloem, and tunneling by high numbers of larvae results in girdling and death of trees. Ash saplings can die after a year of infestation while large trees may die within 3–4 years (Poland, 2007). Since the discovery of EAB in 2002 near Detroit, Michigan, it has spread to contiguous areas of the United States and Canada. Due to the transport of infested ash firewood or nursery stock, however, infestations are now known as far east as Maryland and as far west as Minnesota. As of May 2007, infested areas in Michigan, Ohio, Indiana and Ontario exceeded 103,600 km^2, and over 30 million ash trees had been killed in Michigan alone (http://www.emeraldashborer.info).

The beetle is considered a minor and periodic pest of ash trees in most of its native range (Gao et al., 2004), likely due to the presence of natural enemies and ash species with higher resistance to the beetle (Liu et al., 2003). The potential of natural enemies for biological control of EAB in North America led to further research in China, within the beetle's native range, on the population biology of EAB and its parasitoids on different ash species (Bauer et al., 2005, 2006; Liu et al., 2007). Surveys of natural enemies attacking beetle populations in North America, primarily in Michigan where the beetle is most widespread, were also conducted (Bauer et al., 2004, 2005). The results of research conducted from 2002 to 2004 in southeastern Michigan revealed <1% of EAB were parasitized by braconids, a chalcid and a eupelmid (Bauer et al., 2005). Approximately 2% were infected with fungi, including isolates of *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Isaria farinosa* (Holmsk.) Fries (formerly *Paecilomyces farinosus*), *I. fumosorosea* Wize (formerly *P. fumosoroseus*), and *Lecanicillium lecanii* (Zimmerman) Viegas (Bauer et al., 2004, 2005). Further sampling by L.S. Bauer and

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H. Liu in different areas of Michigan resulted in additional *B. bassiana* isolates from infected EAB (unpublished data). These isolates represent some of the presumably indigenous fungal populations pathogenic to the exotic EAB.

The objectives for our study were to: (1) determine the reservoir for *B. bassiana* by culturing fungi from soil and bark sampled from ash trees at Michigan field sites where infected beetles were previously collected; (2) characterize and compare the *B. bassiana* isolates obtained from infected EAB and the bark and soil samples; and (3) compare the virulence of representative *B. bassiana* isolates against adult EAB. These data will provide information on the potential of indigenous fungal strains for use in the microbial control of EAB and may demonstrate effective strategies for their deployment in the field.

2. Materials and methods

2.1. Sample collection and fungal isolation

Beauveria bassiana was isolated from EAB larvae and pre-pupae from infested ash trees felled in Michigan field sites from 2002 to 2006. Infected beetles were observed during dissection and rearing out of adults from infested logs in the laboratory. Some of the field sites sampled were part of earlier research on EAB natural enemies, initiated in 2002 (Bauer et al., 2004, 2005). Many of these fungal isolates were deposited in the USDA ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, NY) (LSB and HL, unpublished results).

In our present study, we obtained 42 of the EAB-derived *B. bassiana* isolates from ARSEF, established monosporic isolates (Castrillo et al., 2004), and assessed their molecular diversity. To reveal possible reservoir of *B. bassiana* inocula, we returned to four of the previously-studied field sites in September 2007 and sampled soil and bark from some remaining stumps and dead trees, primarily green ash, *F. pennsylvanica* Marsh. There were numerous EAB exit holes and old galleries under the bark of these ash trees, indicating previous EAB infestation. The four sites were Hudson Mills Metropark in Dexter, Island Lake Recreation Area in Brighton, Western Golf and Country Club in Redford, and Willoughby City Park in Lansing. The number of bark and soil samples collected at each site are listed in Table 1. When these sites were sampled earlier, EAB densities were moderate (50–100 EAB/m^2) at Brighton, and high (>100 EAB/m^2) at Dexter, Redford, and Lansing (LSB and HL, unpublished data). *B. bassiana* was isolated from bark and from soil samples collected at the four sites and compared to EAB-derived isolates. These isolates and those sampled from infected beetles were identified as *B. bassiana* based on morphological characters (Humber, 1997).

Ash bark (~3.6 cm^2) was sampled from four to five remaining ash trees or stumps in each site by use of a wood chisel (1.9 cm) that was disinfected with 10% bleach between samples. Each sample was collected in a sterile 50-ml conical polypropylene tube, transported in a cooler and stored at 4 °C until processing within a few days. Soil samples were collected from the upper 8 cm of soil surface beneath each sample tree using a soil auger (1.6 cm diam.) (Oakfield Apparatus Company, Oakfield, WI, USA) that was also disinfected with 10% bleach between samples. Additional soil samples were collected in Redford from mounds where infested ash trees were felled. Each soil sample was stored in a sterile sampling bag and handled as the bark samples. The bark samples were vortexed in 10 ml of autoclaved 0.2% aqueous Tween 80 (Fisher Scientific, Suwanee, GA), a 10-fold dilution was prepared and 200-μl aliquots plated on wheat germ dodine agar (WGDA; Sneh, 1991), a semi-selective medium. For soil samples, 10 g subsamples were transferred to sterile 50-ml conical polypropylene tubes and vortexed in 9 ml of 0.2% aqueous Tween 80. The soil suspension was held stationary for 5–10 min to allow large particles to settle before the supernatant was subjected to a series of three 10-fold serial dilutions, after which 200-μl aliquots were plated on WGDA. We used three replicate plates per stock wash and dilutions per bark or soil sample. Plates were incubated at 24 °C with 16:8 L:D and examined after 5-7 days.

Representative colonies with *Beauveria* sp. morphology were transferred to Sabouraud dextrose agar plates supplemented with 1% yeast extract (SDAY; Difco Manual) and incubated under the same conditions used for WGDA plates. Monosporic isolates were established from *B. bassiana* colonies representative of the different morphotypes detected. A total of 21 soil-derived and 15 bark-derived isolates were included in this study. We included three additional soil-derived isolates collected in 2006 at Gee Farms Nursery, Stockbridge, Michigan, where we evaluated the field efficacy of the *B. bassiana* commercial strain GHA. These three represented the more common genotypes collected from this site. We also included the commercial strain GHA, which we isolated from a technical grade spore product (lot 980528) provided by Mycotech (now Laverlam International Corp., Butte, MT). Throughout this paper, we will refer to each fungal collection as an isolate, whereas isolates will be referred to as strains if they have been characterized by molecular assays in other studies or later in this study (Carlile et al., 2001). Following molecular analysis, we deposited representative strains derived from the bark and soil samples to ARSEF. The fungal strains used in this study are listed in Fig. 1.

2.2. Molecular characterization of fungal isolates

Fungal DNA was extracted from blastospores, grown as described by Pfeifer and Khachatourians (1993), using the DNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol with modifications. Blastospore pellets from 2 ml of 4-day-old cultures were homogenized with 0.5 g of 0.5 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) in 400 μl of lysing buffer (Qiagen) using a Mini Bead Beater (BioSpec Products) for 30 s at 4200 rpm. DNA was eluted once with 100 μl elution buffer (Qiagen) and stored at −20 °C until used.

Seven microsatellite markers developed by Rehner and Buckley (2003) for *B. bassiana* were used to assess genetic diversity among the beetle-, bark-, and soil-derived isolates. The primers (GenBank accession No.) used were: Ba01 (AY212020), Ba02 (AY212021), Ba03 (AY212022), Ba06 (AY212024), Ba08 (AY212025), Ba12 (AY212026), and Ba13 (AY212027). PCR assay conditions were as reported in Castrillo et al. (2008). Assays were repeated at least twice for each primer-isolate pair. PCR products were visualized in 3% (wt/vol) Metaphor gel (Lonza, Rockland, ME) in TBE buffer (90 mM Tris-borate, 2.0 mM EDTA), stained with ethidium bromide. Gels were analyzed using GeneGenius gel documentation and imaging system (Syngene, Frederick, MD).

The molecular size of each PCR product generated by each microsatellite primer pair was calculated using Gene Tools (Syngene).

Table 1
Beauveria bassiana colony forming units (CFU) obtained from ash bark and soil samples from different sites in Michigan.

Collection site	Mean *B. bassiana* CFU ± SE (samples with CFU/total collected)	
	per 3.6 cm^2 bark sample	per 10 g soil sample
Brighton	0 (0/5)	0 (0/5)
Dexter	$1.9 \pm 2.78 \times 10^2$ (2/5)	189 ± 135 (4/5)
Lansing	0 (0/5)	$7.85 \pm 9.89 \times 10^3$ (5/5)
Redford	$3.25 \pm 5.74 \times 10^3$ (3/4)	20 ± 40 (2/8) [a]

[a] Four of the eight soil samples from Redford, MI, were collected from mounds where infested ash trees were felled. None of these samples yielded any *B. bassiana* CFU. The other four samples were collected at the base of ash trees.

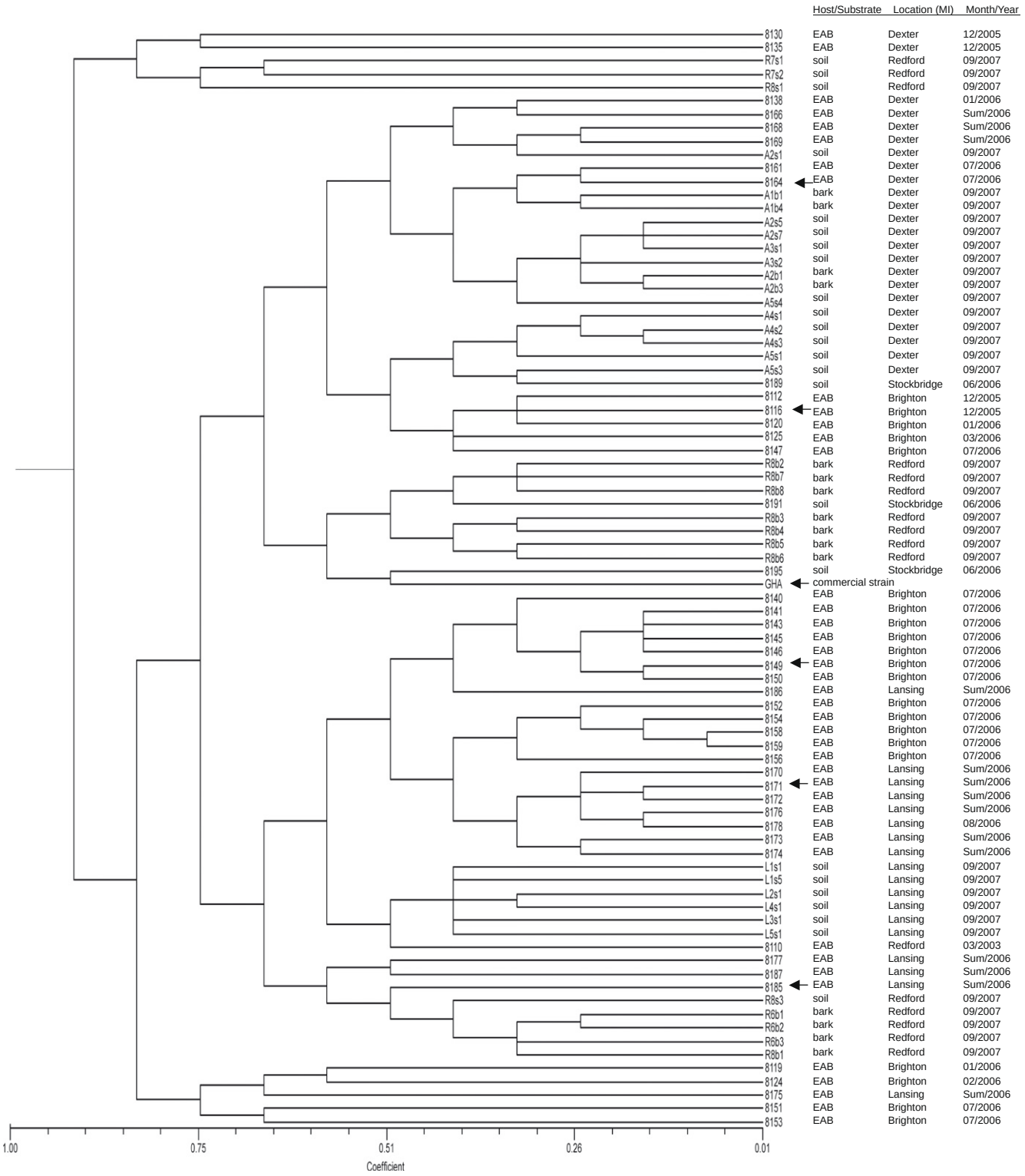

Fig. 1. Dendrogram representing genetic dissimilarity based on Dice coefficient among 81 strains of *Beauveria bassiana* isolated from the emerald ash borer (EAB), green ash trees and soil in Michigan, and the commercial strain GHA. Coefficients were based on a total of 132 alleles detected with seven microsatellite primers. Cluster analysis was performed using the UPGMA method and consensus tree was computed using the strict consensus method. All EAB-derived strains have four-digit numerical ARSEF codes; bark and soil-derived strains, except those from Stockbridge, have mixed letter-number codes. Collection month or season (Sum, summer) and year are listed for each strain. Strains used in EAB adult bioassay (Table 2) are marked with an arrow.

Each unique DNA fragment was assigned an allele number by microsatellite locus and scored as present or absent for each isolate. The data were analyzed using the NTSYS-pc program version 2.2 (Exeter Software, Setauket, NY). A similarity matrix was calculated using the Dice coefficient, recommended as the suitable measure of similarity for haploid organisms with codominant markers (i.e., microsatellite markers) (Kosman and Leonard, 2005). Cluster analysis was performed using the unweighted pair-group method using

arithmetic averages (UPGMA) and a consensus tree was computed using the strict consensus method (Sneath and Sokal, 1973).

2.3. Comparative virulence of representative strains

Comparative virulence of representative EAB-derived strains was determined by bioassays of adult beetles. Adult EAB were reared from infested ash trees according to the methods described by Liu and Bauer (2006). For the bioassays, we used 3- to 4-day-old adults maintained in the laboratory on greenhouse-grown *F. uhdei* (Wenzig) Lingelsh foliage, which was changed every 2 days (Liu and Bauer, 2006).

EAB-derived *B. bassiana* strains ARSEF 8116, 8149, 8164, 8171, and 8185, each representative of different genetic clusters (Fig. 1), were selected for bioassay studies and compared with the commercial strain GHA. Dried conidia were produced for all strains following protocols reported in Castrillo et al. (2008). Viability of fungal stocks was estimated by percentage germination of conidial suspensions on half-strength SDAY plates incubated at 24 ± 1 °C and 16:8 L:D for 16–18 h. All strains had >95% viability prior to bioassays. Stock suspensions of 25 mg conidia/15 ml of aqueous 0.01% Tween 80 and 1 g of 2 mm glass beads (BioSpec Products) were prepared in 50-ml polypropylene centrifuge tubes and agitated on a wrist-action shaker (Model BT, Burell Scientific, Inc., Pittsburg, PA) set at a maximum speed of 6.7 oscillations/s for 15 min. Conidial concentrations were estimated using a Neubauer hemocytometer. A four series 10-fold dilution from 3.6×10^7 conidia/ml was prepared for GHA and 3.6×10^6 conidia/ml for the EAB-derived strains. However, technical problems encountered during the first assay resulted in a lower initial stock of 2×10^7 conidia/ml for GHA. For the second assay we used the same dosages as applied in the first assay.

For each assay, 30 EAB adults were dipped individually in a fungal suspension three times in quick succession, blotted on paper toweling to remove excess moisture and transferred to individual petri dishes (100 × 15 mm) containing a *F. uhdei* leaflet inserted in a 2-ml microcentrifuge tube with a punctured lid and filled with 1.5 ml water. Assay dishes were held at room temperature (22 ± 2 °C) with 15:9 L:D and checked daily for insect mortality for 6 days. Ash leaves were replaced every 2–3 days. Dead beetles were removed from assay dishes, transferred to WGDA plates and examined for sporulation after 7 days. Sixty control beetles were dipped in 0.01% Tween 80 and handled as described above.

2.4. Statistical analyses

Mortality data from *B. bassiana*-treated beetles after 6 days were corrected for control mortality using Abbott's formula (Abbott, 1925). The dose–response of EAB to GHA was analyzed by probit analysis (LeOra Software, 1987). Using the resulting equation, we estimated mortality from GHA inoculation at 3.6×10^6 conidia/ml, the dose used for the EAB-derived strains. The variance of arsine-square root transformed percentage mortality was analyzed among isolates and compared to GHA using Dunnett's test. Survival analysis with the Weibull function was used to estimate the time (hours) to 50% mortality (ST_{50}) for each isolate and dosage (JMP Software, 2007).

3. Results

3.1. Collection of B. bassiana isolates

The number of *B. bassiana* colony forming units (CFU) observed from bark and soil samples was variable, ranging from 0 to 1.2×10^4 CFU/3.6 cm^2 bark sample from tree stumps in Redford and 9.8×10^2 to 2.4×10^4 CFU/10 g soil sample from a woodlot in Lansing (Table 1). Comparison of CFU obtained from tree bark with adjacent soil core showed that more sample pairs had CFU only in the soil. Of the 19 bark-soil pairs collected, eight had *B. bassiana* CFU only in the soil sample, two had CFU only in the bark sample, three had CFU in both, and six yielded no *B. bassiana*. The two bark samples that had CFU where none was found in the soil were from Dexter (3.3×10^2 CFU/sample) and from Redford (1.2×10^4 CFU/sample).

3.2. Molecular characterization of B. bassiana isolates

Characterization of EAB-derived *B. bassiana* strains from the four Michigan field sites by use of seven microsatellite markers revealed high genetic diversity (Fig. 1). Of the 42 EAB-derived strains, only four clonal groups, each comprised of two to four strains collected from the same site at the same time, were observed (not shown on consensus tree). A total of 32 EAB-derived strains with unique genotypes were found. Our strategy to sample most of the detectable morphotypes likely minimized clonal samples and the determination of more prevalent genotypes. However, this strategy maximized the detection of genetic diversity among *B. bassiana* strains associated with EAB.

Our *B. bassiana* genetic diversity data revealed that most of the EAB-derived strains from Lansing and from Dexter clustered with soil- or bark- and soil-derived strains, respectively, from the same site. EAB-derived strains from Brighton, which were the most numerous samples, were found in three clusters: one set of five strains grouped with strains from Dexter and Stockbridge soil samples; a set of 12 strains grouped with strains from Lansing soil samples; and a set of four strains with less than 25% similarity to the other two sets (Fig. 1). The absence of isolates from Brighton soil and ash bark did not permit comparison of genotypes from beetle- versus bark- or soil-derived strains from this location. Within location, the mean genetic similarity of EAB-derived strains from Dexter, Brighton and Lansing was 27%, 26% and 19%, respectively. Only one EAB-derived strain was available from the Redford sampling site.

Comparison of haplotypes detected among bark and soil samples from Dexter and from Redford, the only two sites where both bark and soil sample pairs yielded *B. bassiana* CFU, showed that while all Dexter strains clustered together, Redford strains were found in different clusters. Most of the Redford bark-derived strains showed more similarity to a strain from Stockbridge soil than to strains from Redford soil. Comparison of the commercial strain GHA with EAB-associated or indigenous Michigan strains showed that it is unique, indicating that GHA is not native to the sites tested, nor has there been any GHA-based mycoinsecticide application in or near the sites sampled.

Overall, a total of 132 alleles were detected from the seven microsatellite loci in the 82 strains of *B. bassiana* tested. The number of alleles and their size range (base pair or bp) detected by each primer were: Ba01 (19, 85–111 bp); Ba02 (15, 109–153 bp); Ba03 (20, 119–197 bp); Ba06 (20, 88–130 bp); Ba08 (29, 187–282 bp); Ba12 (17, 117–133 bp); and Ba13 (12, 140–203 bp).

3.3. Virulence against EAB adults

After 6 days the mean mortality among control beetles was 18 ± 3% (n = 60/assay). None of the dead control beetles showed evidence of fungal infection, indicating the absence of background fungal contamination or infection. The probit-transformed mortality varied with $\log_{10}$ dosage of GHA (slope = 0.6; SE = 0.1; χ^2 = 0.86; df = 4; $P > 0.1$; $LC_{50} = 3.1 \times 10^4$; 95% fiducial limits = $4.5 \times 10^3 - 9.4 \times 10^4$). Dunnett's test showed no significant difference among angular-transformed mortalities for indigenous

strains and GHA ($|d| = 3.62$; $P < 0.05$; df = 5). The estimated GHA mortality at 3.6×10^6 conidia/ml, the same dosage used for EAB-derived strains, was 90 ± 12%. Mortality values for strains 8116, 8149, 8164, 8171, and 8185 were 73 ± 10%, 92 ± 2%, 73 ± 4%, 90 ± 4%, and 84 ± 2%, respectively.

Analysis of ST_{50}s showed overlapping 95 percent confidence intervals for most strains (Table 2). Because of the large degree of overlap among strains, we did not do further analysis. The lowest average survival time was 96 h for beetles treated with the highest dosage of GHA. The highest average survival times (134 and 129 h) were for beetles treated with strain 8164 and 8116, respectively.

4. Discussion

Characterization of *B. bassiana* strains associated with EAB in Michigan has shown a genetically diverse group mostly of indigenous origins. Comparisons of EAB-derived strains versus soil- and bark-derived strains indicate that the soil serves as the primary source of fungal inocula that may be dispersed to tree trunks with rain splash or by air currents as reported for other hypomycetous fungi like *Hirsutella cryptosclerotium* Fernandez-Garcia, Evans & Samson and *Nomuraea rileyi* (Farlow) Samson (Fernandez-Garcia and Fitt, 1993; Garcia and Ignoffo, 1977). In cases where CFU were obtained from both bark and the adjacent soil, strain genotypes from both samples were found similar or the strains grouped in the same cluster. A few cases were observed, however, where only bark samples yielded *B. bassiana* CFU. These few samples suggest that in addition to the soil fungal reservoir, infected EAB or other insects infesting ash trees such as trunk borers and bark beetles (Solomon et al., 1993) could serve as sources of inocula and could move inocula from one site to another. Natural dispersal of infected EAB or movement of infested nursery stock or firewood with infected beetles could explain the presence of a few EAB-derived strains from Dexter with higher similarity to soil strains from Redford than to the majority of strains collected from Dexter. Avian dispersal of fungal inoculum between strands of trees is also possible, with woodpeckers in particular spending much time foraging on EAB-infested ash trees (Lindell et al., 2008).

For EAB populations, dispersal of indigenous *B. bassiana* propagules, possibly by air current, rain splash, or infected beetles, onto ash trunks limits widespread dispersal and heavy inocula build up that could trigger epizootics of the fungus. EAB adults feed on ash foliage throughout their lives. However, they spend considerable time in contact with ash bark during emergence when they chew through the tree bark, when mating and searching for oviposition sites, and during egg deposition between bark layers and crevices (Liu and Bauer, 2006, 2008a,b). Early in the infestation cycle, when beetles first arrive, they generally attack healthy ash trees along the upper trunk (LSB and HL, unpublished data). Thus, early in an infestation, EAB adults may be less likely to encounter fungal inocula. During the larval stage, neonates may become inoculated when boring through the bark to reach the phloem. In addition, the bark often splits over the larval gallery due to callous formation, and fungal inocula can readily enter the gallery and infect larvae and pre-pupae (Liu and Bauer, 2008a,b). This was exemplified within our fungal collection, with all of the strains collected from larvae or pre-pupae. Our collection may also reflect a sampling bias because larvae are easier to collect than the more mobile adults, and infected adults are harder to find in the field. In an earlier bioassay, however, we found that adult EAB were more susceptible to fungal infection than larvae or pre-pupae (LSB and HL, unpublished data).

Comparative virulence studies of five EAB-derived strains from different genetic clusters showed that all five were similar in virulence to the commercial strain GHA. Our bioassay studies, however, had relatively high mortality among control beetles. We suspect that this may be due to the use of adults kept in logs at 4 °C and only allowed to emerge months past their normal emergence time in June. This method of obtaining adults has its limitations and further studies need to be conducted to develop an artificial diet for rearing EAB. Nonetheless, these bioassay data suggest that virulence is not a limiting factor in the development of fungal epizootics, especially in heavily infested areas, but that the mode of inocula pick up and level of inocula present on ash trunks constrain infection prevalence in EAB populations. Although the soil could serve as a reservoir of fungal inocula that could be transferred to ash trunks, the inocula remaining on ash bark would provide a more accessible source for beetle infection. Ash trunks, thus, could serve as an important fungal reservoir that could be used to target both EAB adults and larvae. Current field control strategies of applying GHA-based mycoinsecticide to the bark prior to beetle emergence could prove viable since this strain is virulent to EAB (Liu and Bauer, 2006, 2008a,b) and also persists for several weeks on ash bark (Castrillo et al., 2010). Thorough coverage of ash trunks with fungal inoculum obviates the constraints of low or uneven fungal distribution, and persistence of sufficient inocula on bark could target not only emerging adults but also ovipositing females and eclosing larvae. Indeed, Liu and Bauer (2008a) have shown improved field efficacy of GHA when sprayed to trunks prior to adult beetle emergence. They found infection rates ranging from 58.5–83% in EAB adults in the field. They also observed that infection rates in larvae were positively correlated to larval density in the field, further demonstrating the impact of fungal inocula present on ash trunk and the means by which *B. bassiana* can be used to manage EAB populations.

Table 2
Estimated times to 50% mortality of EAB adults exposed to *Beauveria bassiana* commercial strain GHA and five EAB-derived strains.

Strain	Dosage	ST_{50} (h)[a]	95% C. I.
GHA	2×10^4	150	131–172
	2×10^5	123	113–134
	2×10^6	116	109–123
	2×10^7	96	86–105
8116	3.6×10^6	129	120–137
8149	3.6×10^6	110	102–119
8164	3.6×10^6	134	126–142
8171	3.6×10^6	117	110–124
8185	3.6×10^6	114	106–122

[a] Survival analysis with the Weibull function was used to estimate the time (hours) to 50% mortality (ST_{50}) for each strain and dosage. Corrected for control mortality 6 days after inoculation ($n = 60$ beetles for each of two assays).

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